Verwendung von drohnenbrütigen Arbeiterinnen zur Zucht auf individuell exprimierte Merkmale der Honigbiene

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Vice versa

Ein Hase sitzt auf einer Wiese
des Glaubens, niemand sähe diese.

Doch, im Besitze eines Zeißes,
betrachtet voll gehaltnen Fleißes

vom vis-a-vis gelegnen Berg
ein Mensch den kleinen Löffelzwerg.

Ihn aber blickt hinwiederum
Ein Gott von fern an, mild und stumm.

Christian Morgenstern

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Last figure: Use of individual honey bee workers as energy source for enlightenment.

Zusammenfassung

In der Bienenzucht erfolgt die Selektion meist auf Volksebene. Dennoch gibt es einige Merkmale, wie z.B. das Hygieneverhalten, die sich sinnvoll an einzelnen Arbeiterinnen messen lassen. In solchen Fällen kann der Zuchtfortschritt potentiell beschleunigt werden, wenn Söhne der selektierten Arbeiterinnen zur Besamung herangezogen werden. Ziel dieser Arbeit war deshalb die Schaffung von Methoden zur Erzeugung von Söhnen selektierter Arbeitsbienen.

Um die Fruchtbarkeit der selektierten Tiere zu fördern, wurde zunächst die Ernährung optimiert. Dann wurde versucht, eine Hormonbehandlung zur gezielten Induktion der Eibildung zu entwickeln. Dabei konnten die Mechanismen der Fortpflanzungssteuerung bei Arbeitsbienen teilweise aufgeklärt werden. Die Entwicklung einer hormonellen Methode zur Fruchtbarkeits-Induktion war jedoch nicht möglich.

Als Nächstes wurde eine Methode erarbeitet, um die Fruchtbarkeit in allen Bienen außer den selektierten zu unterdrücken, um die Identifizierung von deren Eiern zu ermöglichen und Konkurrenz zwischen selektierten Tieren und Begleitbienen zu verhindern. Dazu wurde zunächst versucht, Arbeitsbienen durch Bestrahlung zu sterilisieren. Die erhaltenen Tiere legten keine Eier, zeigten aber starke somatische Schäden. Die Aufzucht von Arbeiterinnen-Larven in überalterten Völkern ergab überraschender Weise keine sterilen, sondern besonders fruchtbare Tiere. Schließlich gelang es unter Verwendung von Flugbienen als Begleittieren, in Jungbienen in über 60% der Fälle volle Ovarentwicklung zu erzielen. Auch konnten alle erzeugten Eier Jungbienen zugeordnet werden.

Um die Eignung von Arbeiterinnen-gelegten Eiern für Zuchtzwecke zu prüfen, wurden Qualitätsparameter gemessen. Arbeiterinnen-gelegte Eier zeigten sich empfindlicher gegenüber Trockenheit und mechanischer Beschädigung als Königinnen-gelegte Eier. Auch enthielten sie in einigen Fällen signifikant weniger Lipide und Dotterprotein.

Alle in Völker verbrachten Arbeiterinnen-gelegten Eier wurden ausgefressen. Also wurde eine Methode zur Anfütterung von Larven im Brutschrank entwickelt. Die so erzeugten Larven überlebten aber ebenfalls nicht in Völkern. Deshalb wurde ein Verfahren entwickelt, mit dem aus über 50% der eingesetzten Larven durch Brutschrankaufzucht adulte Tiere erhalten werden können.

Ein vorläufiges Protokoll zur Erzeugung von Söhnen individuell selektierter Arbeitsbienen wurde erarbeitet. Es ist nicht vollständig validiert, und auf allen Stufen der Eiproduktion und –aufzucht treten Verluste auf. Dennoch stellen die entwickelten Methoden einen erheblichen Fortschritt auf dem Weg zur Zucht aus Arbeitsbienen dar.

Schlagwörter:

Honigbiene, Fortpflanzung, Zucht, eierlegende Arbeiterinnen, Vitellogenin

Summary

Breeding of honey bees usually involves selection at the colony level. However, some breeding traits, like hygienic behaviour, can also be measured at the scale of the individual worker bee. In these cases, genetic progress can potentially be accelerated by using drones which are sons of the selected individuals. The aim of this study was therefore to create methods for the production of offspring from individually selected worker bees.

To enhance fertility of selected workers, diets were developed that maximize ovary development in groups of queenless bees. An attempt to develop a hormonal treatment to induce worker fertility led to the partial elucidation of endocrine regulation of reproduction in worker honey bees. An endocrine method to trigger ovary development could not be developed.

Methods were tested to suppress fertility in all bees except the selected workers, in order to prevent competition among workers and enable identification of the eggs of selected bees. One method tested to produce sterile workers was the application of ultra-hard X-rays. The treated bees were functionally sterile, but showed severe somatic damage. Next, bees were reared inside over-aged colonies in order to reduce their fertility, but the emerging workers were more fertile instead of less. Finally, it could be shown that forager bees can be used as companion bees to selected workers. This method led to fully developed ovaries in more than 60% of bees representing the selected workers in these tests, who also were the only animals to produce eggs.

The next subtask was the measurement of characteristics linked to viability in eggs from laying workers. Worker-laid eggs showed to be more sensitive to dryness and mechanical damage, and in some cases contained significantly less of two important types of nutrients, vitellin and lipids.

When worker-laid eggs were inserted into queenless or queenright colonies for rearing, they were systematically cannibalised. Therefore, a method was developed to rear egs into living larvae in an incubator. However, the larvae produced in this way were not accepted by colonies either. To circumvent this problem, a protocol was designed and validated that allows to rear worker-derived drone larvae into adults *in vitro*.

The study led to a preliminary protocol for the production of drones that are sons of individually selected worker bees. The protocol is not fully validated, and losses occur at all stages of

egg production and rearing. Nevertheless, it represents an important progress towards the efficient use of worker bees for breeding.

Keywords:

honey bee, reproduction, breeding, laying workers, vitellogenin

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Hpg	hypopharyngeal glands
r	coefficient of relatedness
rH	relative humidity

General Part

1 General introduction

1.1 Potential benefits from the use of worker bees for breeding

Almost all traditional breeding traits of the honey bee (*Apis mellifera*) are measured at the level of colonies, not individual queens, drones or workers. For selective breeding, the queens of the best-performing colonies are used to produce young queens and/or drones. For most traits, like honey yield, gentleness or swarming tendency, there is no alternative to this approach, since the expression of these traits results from complex interactions between colony members and cannot be measured in individual bees. However, there are a number of important characteristics that could potentially be selected for at the level of the individual insect. These include morphometric parameters, longevity, and behaviours for which a genetic specialization exists. Most of these traits are specifically expressed in workers, not in queens or drones. In these cases, sons of the workers who carry the trait can potentially be used for breeding (Harris and Harbo, 1991; Thakur et al., 1997; Oldroyd and Osborne, 1999). Although almost all workers are functionally sterile in colonies that contain a queen and normal amounts of brood (Page and Erickson, 1988), they may become fertile in a queenless situation. As they cannot mate, they exclusively produce unfertilised eggs. Because of the special mechanism of gender determination in Hymenoptera, unfertilised eggs almost always develop into males. The use of drones that are sons of workers that carry the trait(s) of interest potentially offers several advantages:

- Drones develop from unfertilised eggs, meaning that they only carry genes of their mother. If the trait observed in the worker bee is mainly influenced by alleles from the worker's father, these alleles are lost if sons of the worker's mother are used. Sons of the worker bee herself, by contrast, also stay a chance of receiving the favourable alleles that the worker has inherited from her father.
- The coefficient of relatedness for the relationship between the sons of workers with the desired genotype and the workers is 0.5 (relationship of mother to son). The coefficient for the relationship between sons of the queen and the worker bee of interest is only 0.25 (relationship of grandmother to grandson) (figure 1). This means that sons of the selected worker are more likely to receive positive alleles present in the worker's genome. Their use should therefore accelerate breeding progress.

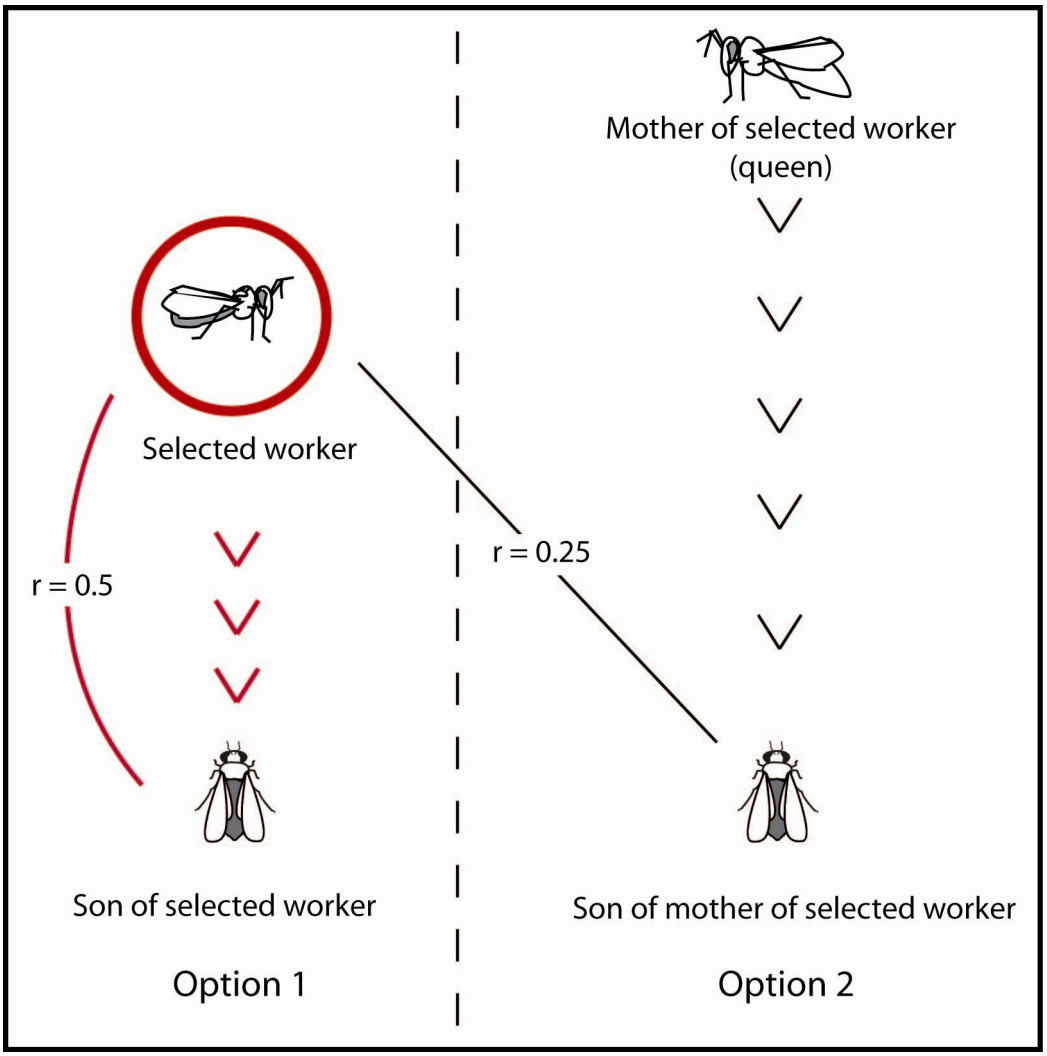

Figure 1: Options for choice of drones after selection at the level of individual workers.

Drones can either be obtained from the selected worker herself or from the mother of the worker (queen of the colony from which the worker was obtained).
r: coefficient of relatedness

As honey bee workers of European subspecies only produce drone eggs, the use of worker offspring is limited to the male side of the breeding scheme.

This study was motivated especially by the prospect of using worker-derived drones for the selection of honey bees for hygienic behaviour. In an apicultural context, the term hygienic behaviour designates the detection and removal of diseased or damaged brood by worker bees (Rothenbuhler, 1964). Hygienic workers are genetic specialists, and bees bred for the behaviour have been found to be less affected by bacterial, fungal and acarian brood diseases (e.g. Spivak and Reuter, 2001, 1998; Basualdo et al., 2008). The trait can be measured at the colony level by introducing damaged or diseased brood and assessing the proportion of emptied

cells after a certain period of time. This technique is used in most breeding programmes, as it is fast and the results have been shown to correlate with the actual breeding objective, disease resistance. A method which allows the assessment of hygienic behaviour of individual workers has been developed by Thakur et al. (1997). Workers are placed on a comb of artificially infested brood, and their behaviour recorded via an infra-red camera. From these recordings, the number of cells opened by each worker can be determined. This number can be used as a measure of the worker's hygienic behaviour (figure 2). In principle, this technique opens the way for the use of worker-derived drones for increasing breeding progress for hygienic behaviour. Breeding from workers for hygienic behaviour may be particularly interesting, because colonies that are hygienic at the colony level are generally rare in populations of European subspecies (Reviewed in Boecking and Spivak, 1999). Colonies that only contain a small number of hygienic workers may not appear to be hygienic, because diseased cells opened by hygienic workers are quickly closed again by "unhygienic" nestmates (Arathi and Spivak, 2001). Selecting at the level of individual workers may offer the precision needed to detect and propagate rare hygienic genotypes (Harris and Harbo, 1991).

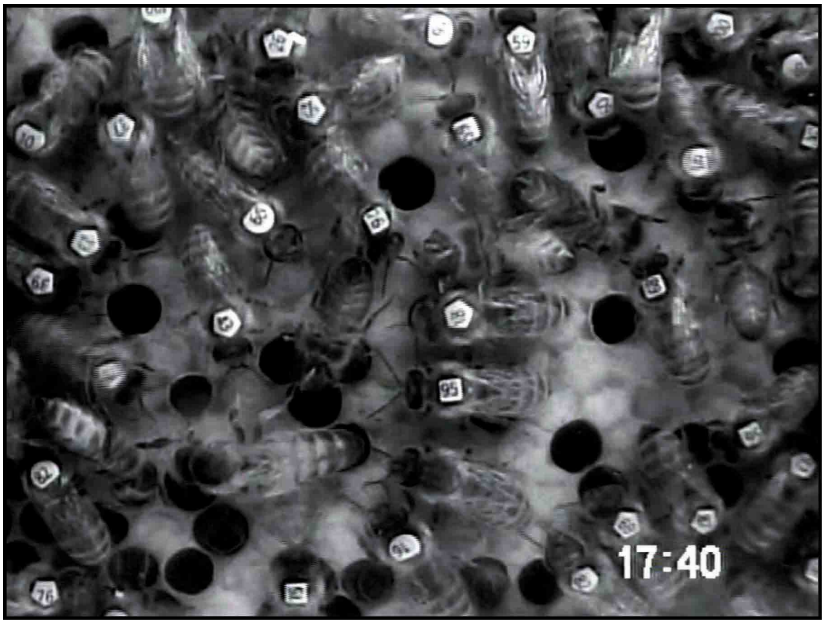

Figure 2: Screenshot from a video that served to measure hygienic behaviour in worker bees.

The cells that appear lighter are capped brood cells, some of which are infested with a brood parasite. Some cells have already been emptied (dark). Each worker carries a numbered tag for identification.
(Courtesy of Fred Zautke, LIB)

1.2 Aims of the study

Despite its potential benefits, the technique of breeding from worker honey bees is hardly used in practice. The reasons for this are likely to be methodological problems. To date, only one protocol has been described that allows the production of eggs from single workers of European descent (Harris and Harbo, 1991), and this method contains major inconveniences (discussed in paragraph 2.2.1). No method has yet been described for rearing worker-laid eggs into adult drones.

The principal aim of the present study was therefore to create protocols for the production of fertile sons of specific, selected worker bees for use in a breeding program

Four subtasks were defined on the way to meeting these objectives (figure 3):

1. Enhance fertility in the selected workers
2. Suppress fertility in the workers accompanying the selected individuals
3. Evaluate the quality of worker-laid eggs
4. Rear worker-laid eggs into fertile drones

Each subtask is the subject of a separate chapter of this thesis. Each chapter starts with a short introduction, followed by summaries of manuscripts related to the subtask, and a discussion of the achievements made.

Work on the four subtasks has led to a total of seven manuscripts. As these were written for a broader public, they present the work mostly from a biological perspective. The significance of the experiments in view of the actual objective of the PhD-study, the development of methods for breeding from worker bees, is not always properly discussed. The summaries in the following sections are therefore intended to place the manuscripts into the context of breeding from worker bees.

Manuscript 1 describes experiments that relate to several subtasks. The different experiments from this manuscript are therefore discussed in different chapters.

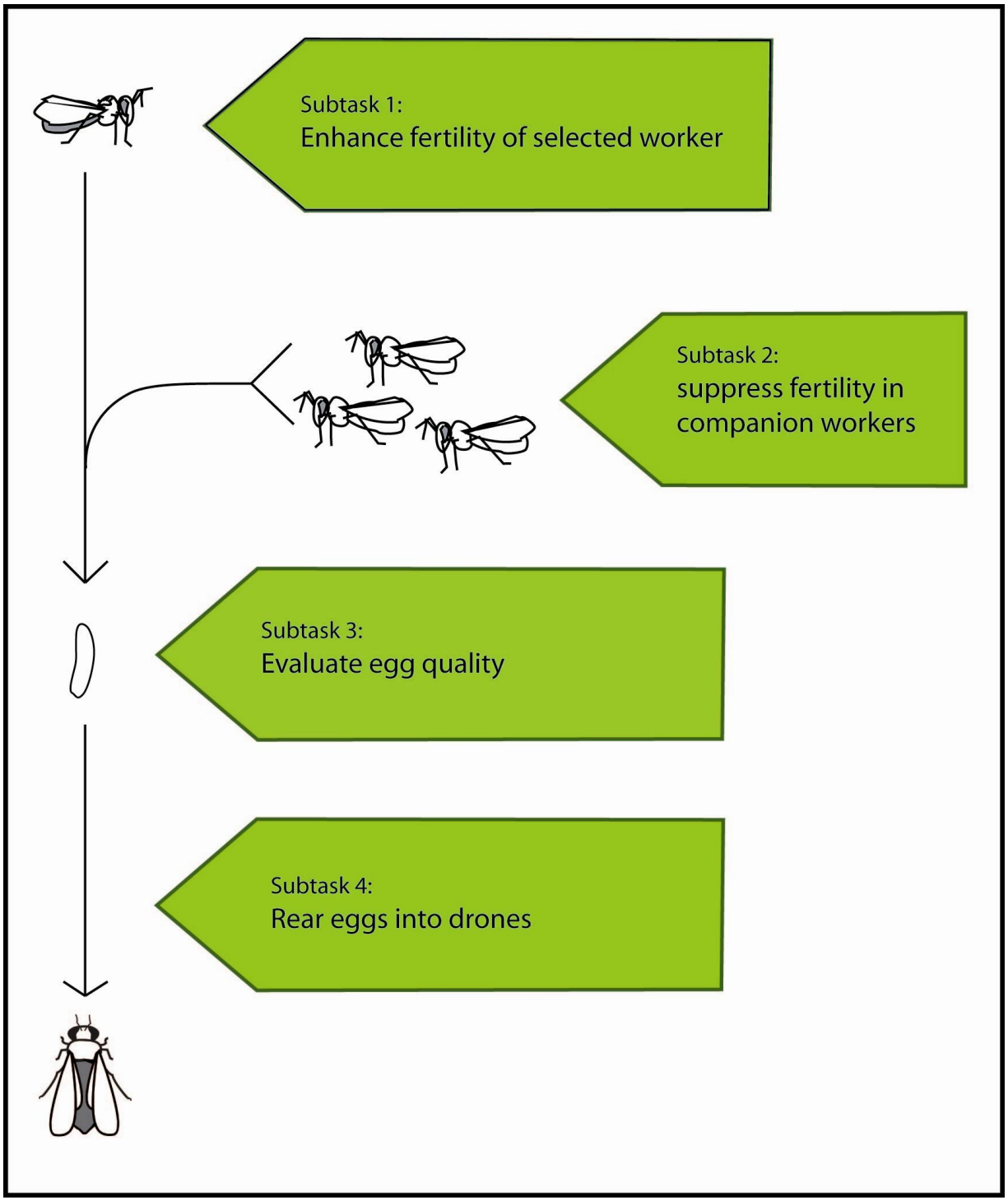

Figure 3: Subtasks of the study. Green arrows show subtasks.

2 Experimental part

2.1 Enhancement of fertility in worker honey bees

2.1.1 Biological background

The first subtask of this study was to find ways to enhance the fertility of selected workers. While it is relatively easy to obtain eggs from groups of 30 or more worker bees held without a queen, to make sure that one or a few particular workers become fertile presents a major challenge. Selection of workers for breeding purposes will usually take place in a situation where workers are queenright, i.e. a queen is present in the colony, and in the presence of brood. Under these circumstances, ovaries in almost all workers are resting (Kropacova and Haslbachova, 1969; figure 4), probably partly because pheromones of the queen and brood suppress worker fertility (reviewed in Le Conte and Hefetz, 2008). Very little worker reproduction takes place in normal queenright colonies, and only one in 10 000 drones produced by such colonies is worker-derived (Page and Erickson, 1988; Visscher, 1989). The first step towards enhancing fertility of selected workers is therefore to isolate them from the queen and brood.

An important factor for the development of fertility in a worker bee is its age at the moment she is separated from the queen (Jay, 1967; Lin et al., 1999), because the physiology of workers is strongly influenced by the task they fulfil, and these tasks follow each other in an age-related manner (reviewed by Robinson and Huang, 1998). The age at which workers can be selected for breeding will depend on the selected trait. For the purpose of this study, it was assumed that selected workers were approximately 7 days old. Bees of this age were used to replace real selected workers in the experiments. Seven days is about the youngest age at which hygienic behaviour can be observed, and one week-old workers have a relatively high chance of developing into fertile animals if they are separated from the queen (Lin et al., 1999).

A second important factor for worker fertility is nutrition. In particular, ovary development in caged groups of worker bees is dependent on a steady supply of protein (Maurizio, 1954; Lin and Winston, 1998; Pernal and Currie, 2000). Optimizing the diet for selected worker bees has therefore been an objective in fulfilling subtask 1.

Finally, the regulation of ovary development in workers strongly depends on social interactions. In queenless groups of bees, a pheromonal contest develops, from which dominant animals emerge that show a higher production of certain pheromonal components otherwise typical of queens (Moritz et al., 2000; Schäfer et al., 2006). In parallel to this pheromonal hierarchy, a feeding hierarchy develops in which dominant workers receive protein-rich glandular secretions (so-called jelly) from subordinate workers by a process called trophallaxis (Korst and Velthuis, 1982; Lin et al., 1999; Schäfer et al., 2006). Most of the proteins contained in jelly stem from the hypopharyngeal glands (hpg, figure 5) of worker bees. As jelly is not only fed to other adult bees but also to developing larvae, the hpg are strongly developed in bees tending to brood (so-called nurses), and usually only weakly developed in the older animals specialized in the collection of nectar or pollen (so-called foragers) (e.g. Lindauer, 1952; Huang and Otis, 1989).

Animals ranking high in the feeding hierarchy are frequently, but not always, identical with those that produce queen-like pheromones. Several studies have shown that in groups of workers where pollen is the only source of protein, it is consumed and digested almost exclusively by non-reproductive workers, who then hand on the protein to reproductive animals in the form of jelly (Lin et al., 1999; Schäfer et al., 2006). Also, the presence of reproductive workers suppresses ovary development in other workers (Velthuis, 1970).

In other social Hymenoptera, it has been shown that the same endocrine signals govern dominance and reproduction, and that both can be induced by injections of certain hormones (Röseler, 1977; Röseler et al., 1984).

In the present study, an attempt was made to develop a hormonal treatment of this kind for the honey bee (manuscript 2).

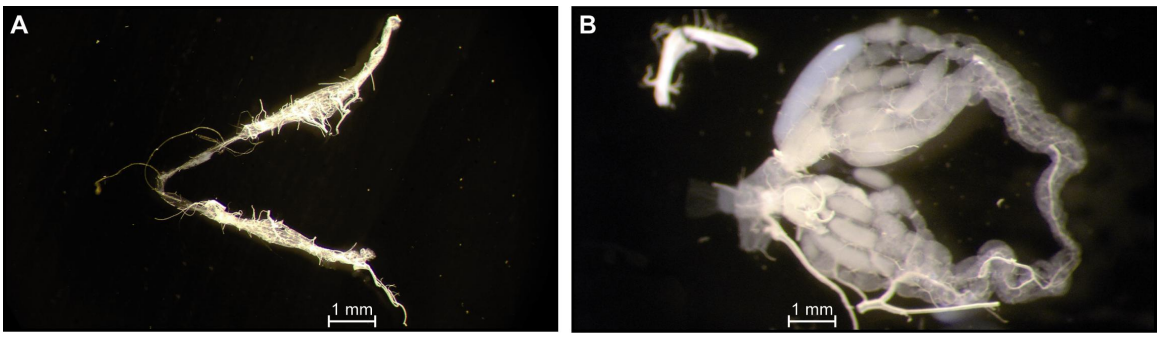

Figure 4: Resting and developed ovaries of worker bees.

A: Resting ovaries, typical of queenright workers.

B: Ovaries of a fertile worker.

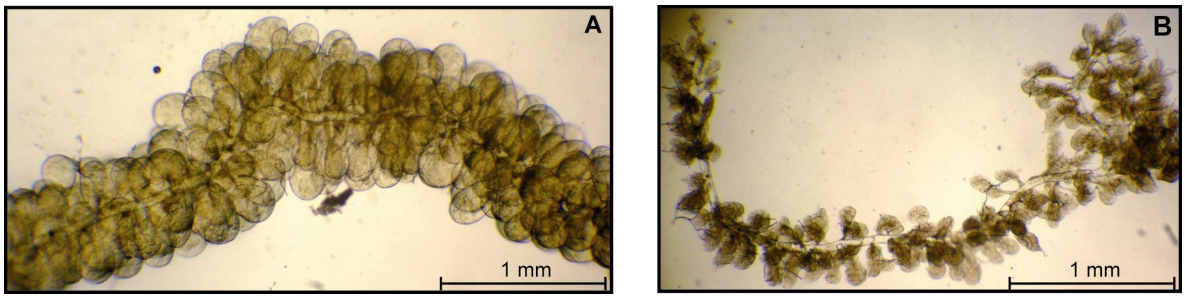

Figure 5: Sections of developed and undeveloped hypopharyngeal glands.

The hpg are paired structures which consist of many glandular lobes, attached to a central duct.

A: Strongly developed.

B: Weakly developed.

2.1.2 Manuscript 1

Wegener, J.; Bienefeld, K.

Methoden zur Zucht der Honigbiene unter Nutzung der Nachkommen von Arbeitsbienen

Submitted for publication in *Züchtungskunde*

Experiment 1: Optimization of a diet to enhance worker fertility

The different experiments described in manuscript 1 relate to different subtasks, and are therefore described in different sections of the thesis. In this paragraph, the first experiment of manuscript 1 is described, which aimed to optimize the nutrition of queenless workers. Groups of bees were placed inside cages and fed diets containing either honey or powdered sucrose as carbohydrate source, and either pollen or royal jelly as source of protein, lipids and other nutritive substances. Ovary development as well as the number, weight and hatching success of eggs produced were monitored for three weeks.

As in an earlier study by Lin and Winston (1998), royal jelly was shown to be a better stimulator of ovary development than pollen, with more than half of the jelly-fed animals presenting fully-developed ovaries after three weeks. However, the greatest number of eggs was obtained from cages supplied with a pollen-containing diet. Egg viability was independent of the

diet fed. It was concluded that both pollen- and royal jelly-containing diets are suitable for supporting worker fertility. Powdered sucrose led to stronger ovary development when mixed to pollen than honey, so it appears to be the better source of carbohydrates. The weight of eggs increased significantly during the study. This suggests that the first eggs obtained from selected workers may not necessarily be the best.

2.1.3 Manuscript 2

Wegener, J.; Huang, Z.Y.; Lorenz, M.W.; Bienefeld, K.

Regulation of hypopharyngeal gland activity and oogenesis in honey bee (Apis *mellifera*) workers

In preparation for publication in *Journal of Insect Physiology*

In order to induce fertility in worker bees, it is essential to understand precisely how ovary development is regulated. It is equally important to understand the mechanisms governing hpg development, because fertility in one worker is thought to depend on the production of jelly by other worker bees (Korst and Velthuis, 1982; Lin et al., 1999; Schäfer et al., 2006). Although the workers that produce jelly and those that produce eggs are believed to be different groups of animals, there is a phenotypic correlation between hpg and ovary development in queenless colonies (Müssbichler, 1952; Nakaoka et al., 2008). Jelly secretion and egg formation are also linked biochemically, because the egg yolk precursor vitellogenin is also a precursor to proteins contained in the jelly (Amdam et al., 2003). One candidate substance for the regulation of vitellogenin uptake by hpg and/or ovaries is the ecdysteroid hormone makisterone. This study aimed at elucidating the regulation of ovaries and hpg, and to explore the possibilities for a makisterone treatment to trigger egg formation.

In a first experiment, groups of young workers were taken from queenright colonies and caged without queens. The sizes of oocytes and of the glandular lobes of hpg, as well as the titre of hemolymph vitellogenin were measured before and at different times after introduction into the cages. For the measurement of vitellogenin, a polyclonal antibody against this protein was custom-prepared.

In the second experiment, hpg activity was suddenly induced by adding brood to previously broodless colonies (hpg-secretions are also fed to developing larvae). The queens of these colonies were caged, so that ovary development occurred as well. Titres of ecdysteroids and

vitellogenin were monitored before and after the addition of brood, using a Radio Immuno Assay.

Experiment 1 showed that hpg and ovary development are correlated in both queenright and queenless workers. The data suggest that both organs may be fundamentally co-regulated. In experiment 2, no reaction of makisterone titres to the activation of hpgs was found. It was concluded that the hormone may not be needed for the induction of jelly secretion. Nearly all animals with makisterone concentrations higher than a particular level possessed oocytes on the brink of vitellogenesis, i.e. the formation of yolk by uptake of vitellogenin. This suggests that a short peak of makisterone may induce vitellogenin uptake by the oocytes. However, it seems unlikely that this effect could be mimicked by an injection of makisterone into workers, since the pre-vitellogenic stages of ovary development probably have to be completed before the hormone can act. The idea of a hormonal treatment for the induction of fertility in selected workers was therefore abandoned.

2.1.4 Achievements

Selected workers have to interact with other workers in order to become fertile, but other workers can also inhibit the selected workers through the development of pheromonal, trophallactic and reproductive dominance (Velthuis, 1970; Korst and Velthuis, 1982). To increase the chance that the selected worker develops into an egg layer, two approaches are possible. Either can the conditions for all workers in the group be improved, so that a greater proportion of the animals become fertile (Maurizio, 1954; Lin and Winston, 1998), or a specific treatment to enhance fertility can be applied only to the selected animal(s).

Approach 1 was followed by optimizing worker nutrition in the cages (experiment 1 of manuscript 1). Through this simple method alone, strong ovary development can be achieved in more than half of the workers in a group if a diet containing royal jelly is fed (although the proportion of ees that actually lay eggs is likely to be much lower). Similar results had already been obtained by Harris and Harbo (1991) with a pollen-containing diet and by Lin and Winston (1998) with a diet based on royal jelly. Eggs produced in the cages showed normal hatching rates, indicating that caging is probably not detrimental to egg quality. Concerns about an increased mortality following feeding of royal jelly (Lin and Winston, 1998) could not be confirmed.

The second approach, targeted enhancement of ovary development in the selected worker, was followed by trying to develop a hormonal treatment with this effect. At the difference to

pheromones, the action of hormones can easily be limited to specific animals. Therefore, they seemed to offer the specificity needed to influence the physiology of particular bees within a group of closely interacting animals. However, the development of such a treatment was complicated by the fact that the hormonal mechanisms controlling reproduction in the honey bee were not fully understood. In most Hymenoptera studied so far, reproduction and dominance are governed at least in part by juvenile hormone, but this is not the case in *Apis mellifera* (reviewed in Robinson and Vargo, 1997). Ecdysteroids, which are a second group of hormones frequently involved in insect fertility, have been suggested to play a role in honey bee fertility (Robinson et al., 1991). The experiments described in manuscript 2 showed that this role may consist in the initiation of yolk uptake by developing oocytes. However, as the time window for ecdysteroid action on ovary development is probably very small, the project of using these substances for fertility enhancement was abandoned. Dombroski et al. (2003) and Sasaki and Harano (2007) have recently shown that feeding and/or injection of certain neurohormones can promote ovary development in worker bees. These results may guide future attempts to develop a hormonal treatment to induce worker fertility.

In summary, the efforts undertaken to enhance the fertility of selected workers produced interesting results from a biological perspective, but did not lead to significant progress with regard to the problem of obtaining eggs from selected workers. Luckily, as will be discussed in the next section, egg-laying by selected workers might frequently be achievable without specific enhancement of fertility.

2.2 Suppression of fertility in the workers accompanying the selected bees

2.2.1 Biological background

Honey bee workers held in isolation show high hemolymph titres of juvenile hormone (Huang and Robinson, 1992), and the juvenile hormone titre in adult bees is negatively correlated with fertility (Robinson et al., 1992). Therefore, selected workers have to be kept in groups with other bees (called “companion workers” hereafter) in order to achieve fertility. Subtask two of this study consisted in producing sterile or near-sterile workers to be used as companion workers. Infertility of companions seemed desirable for three reasons:

- Animals with developing ovaries are recognized (Visscher and Dukas, 1995) and physically attacked by nestmates (Sakagami, 1954: van der Blom, 1991). These attacks rarely lead to injuries, but the victims are forced to give away considerable quantities of food (van de Blom, 1991). The attacking bees are thought to be individuals who seek to become reproductives themselves. Using accompanying bees with a reduced reproductive potential may help avoid competition between them and the selected workers.
- The presence of reproductive workers inhibits the development of ovaries in other workers (Velthuis, 1970)
- Sterile accompanying bees would mean that the parentage of eggs produced in the groups of bees would be clear.

In the only study published to date that deals with methods for breeding from worker bees (Harris and Harbo, 1991), workers from different colonies and breeding lines were screened for their speed of ovary activation after removal from the hive. Either one or five young workers were then taken from the colonies in which worker ovary development had been found to be fastest, and placed in the company of 25 workers from the colony that contained workers in which ovary development was slowest. With this method, eggs were obtained from up to 53% of the fast-developing workers. However, this technique presents several inconveniences. Firstly, bees with interesting traits for breeding may not always be stemming from colonies whose workers show rapid ovary development. Secondly, screening of colonies for fast or slow ovary development in workers is time- and work-intensive. Moreover, the accompanying bees used by Harris and Harbo (1991) were homozygous for a rare recessive colour mutation ("cordovan"), which can only be maintained in highly inbred lines.

Generally, the use of companion bees that are genetically different from the selected workers can be interesting for two reasons. Firstly, if the two strains of bees can be distinguished visually, as in the experiment of Harris and Harbo (1991), this fact may serve to assess the maternity of drones produced. At the Länderinstitut für Bienenkunde (Hohen Neuendorf), where the present study was performed, a strain of yellowish *A.m.Ligustica* has been held for several years for the sole purpose of producing companions to selected workers of the grey subspecies *A.m. carnica*. Secondly, genetical differences relating to worker fertility could be used to favour selected workers, or reduce the risk of egg-laying by companion bees. Different subspecies of the honey bee differ greatly as to worker fertility (Ruttner and Hesse, 1981). Unfor-

tunately, the two most frequently bred subspecies of *A. mellifera*, *A.m. ligustica* and *A.m.carnica*, are both characterized by relatively slow ovary development in workers (Ruttner and Hesse, 1981). Also, any genetic solution to the problem of producing sterile companions would require important investments of time and other resources. Therefore, other approaches were followed in the present study.

2.2.2 Manuscript 3

Wegener, J.; Zautke, F.; Hoecht, S.; Köhler, B.; Bienefeld, K. (2006)

Suppression of fertility in the worker honey bee (Apis mellifera) by treatment with X-rays.

Journal of Apicultural Research 45: 27-32.

The objective of this work was to produce sterile companion bees by irradiation, using methods similar to those applied for the Sterile Insect Technique in pest control (e.g. Bakri et al., 2005). Radiation damage mainly concerns dividing cells (Nias and Dimbleby, 1990). Cells of the ovary and germ line of female bees mostly form before the completion of larval stage 3 (Reginato and Cruz-Landim, 2002), but insect larvae are unsuitable for sterilization by irradiation, because cells in their somatic tissues are dividing, too. Therefore, irradiation had to be done as early as necessary in order to achieve sterilization, but as late as possible in order to minimize somatic damage.

In order to determine the optimum age at irradiation, brood of different stages was treated with a standard dose of hard X-rays. Ovary and hpg development, as well as egg-laying were monitored in caged groups of workers emerging from the irradiated brood. The results were compared to those obtained with untreated controls.

Workers emerging from brood irradiated at all the stages included in the experiment showed suppression of egg production for at least 21 days, the duration of the experiment. However, mortality in all irradiated groups was heightened as compared to controls, particularly if the brood was irradiated before a particular stage known to mark the completion of mitotic divisions in relation to metamorphosis (Oertel, 1930). All irradiated animals showed very low levels of hpg development and strong signs of somatic damage. It was therefore concluded that, at least at the dosage tested, irradiation is probably not a good technique for producing sterile or subfertile companion bees.

A useful by-product of this study was the development of special cages that simplify the monitoring of oviposition and the harvesting of eggs (figure 1 in manuscript 3).

2.2.3 Manuscript 4

Wegener, J; Lorenz, M.W.; Bienefeld, K.

Physiological consequences of prolonged nursing in the honey bee.

Accepted for publication in *Insectes Sociaux*

It has been shown repeatedly that the fertility of worker honey bees is influenced by the conditions under which they are reared as larvae (von Rhein, 1933; Maurizio, 1954; Hoover et al., 2006). Haydak (1963) has reported that workers reared by over-aged nurses weigh far less than normal workers, and weight and fertility are usually positively correlated in bees (Hoover et al., 2006; Akyol et al., 2008). It was therefore expected that rearing by old nurses would lead to workers of low fertility, which might be used as companion bees.

Two groups of queenright experimental colonies were prepared. Colonies of one age group had a near-normal age structure, while all bees in the second group were older than 23 days. All colonies received a similar number of eggs, which had all been laid by the same queen. Nurse bees that were seen to tend the larvae emerging from these eggs were sampled for measurements of physiological parameters. Workers reared in both types of colonies were transferred into a queenless colony in order to evaluate their fertility.

The only significant difference between the nurses from over-aged and regular colonies concerned the mandibular glands, which mainly produce the lipoid fraction of brood food. As expected, workers reared in the over-aged colonies were lighter, which may either be a consequence of nurse age or of the smaller numbers of attending nurse bees left in these hives. Surprisingly, workers reared in over-aged colonies also showed a stronger degree of ovary development and a higher number of ovarioles than bees from regular colonies. This suggests that qualitative differences exist between the brood food produced by regular and over-aged nurses. In any case, rearing by old nurses is not a suitable method to produce companion bees of low fertility.

2.2.4 Manuscript 1 (continued)

Experiment 2:

Use of old hive bees or forager bees as companions to selected workers

Fertility is usually highest in workers that are young nurses at the time the queen is lost, and low in old bees, particularly in foragers (Dreischer, 1956; Lin et al., 1999; Patricio and Cruz-Landim, 2003). This second experiment of manuscript 1 aimed to test the suitability of old bees as companions to selected workers.

Cages were prepared that contained 30 of the companion bees to be tested, plus three 7 day-old bees (representing the selected workers). Two types of "old" bees were tested. In a first round of the experiment, "old" bees were produced by repeatedly removing all the brood from small colonies, so that no young workers hatched. In a second round of the experiment, "old" bees were foragers.

The groups of animals received a pollen-containing diet (manuscript 1, experiment 1). All eggs laid were frozen and stored. Four days after egglaying had started in a cage, the cage was frozen and the degree of ovary development was determined in all bees. In case both young and companion bees were found to present strongly developed ovaries, the eggs and the potential mothers were genotyped by microsatellite analysis in order to verify egg parentage. These analyses were performed by staff of the Institut für Fortpflanzung landwirtschaftlicher Nutztiere e.V., Schönow.

In both rounds of the experiment, less than 3% of companion bees had fully developed ovaries at the moment the cages were frozen, compared to more than two thirds of the 7 day-old workers. In round one, eggs were produced in 7 cages out of 9. However, egg-laying by companion bees occurred in two cages out of these 7. In round two (companion bees = foragers), eggs were found in 15 out of 23 cages, and all eggs found had definitely been laid by young workers. These results show that fertility in specific workers can be selectively induced by association with older bees. Foragers are far easier to obtain than over-aged hive bees, and their fertility seems to be even lower. Therefore, foragers may be more suitable for use as companion bees than over-aged hive bees.

2.2.5 Achievements

It had been shown that queenless workers with developed ovaries receive most of their protein through trophallaxis (Lin et al., 1999; Schäfer et al., 2006). Therefore, manuscripts 2 - 4 aimed at the production of companion bees that were not only sterile, but also presented strongly developed hpgs. Unfortunately, bees of the age group in which hpg development peaks (5 to 15 days; Lindauer, 1952; Huang and Otis, 1989; Hrassnigg and Crailsheim, 1989) also have the greatest chance of becoming egg-layers if placed in a queenless situation (Lin et al., 1999). All our efforts to solve this dilemma were in vain. Targeted destruction of reproductive tissues by irradiation failed (manuscript 3). The production of sterile young bees by rearing in over-aged colonies also failed, leading even to particularly fertile adults (manuscript 4). The use of over-aged hive bees as companions (1st round of experiment 2, manuscript 1) was only a partial success, because not all of them turned out to be functionally sterile.

A new approach to the problem of producing sterile companion bees was motivated by circumstantial observations. During dissections of queenless workers from cages that had received a pollen-containing diet, it was noticed that many fertile bees presented great amounts of pollen remainders in their recta. This suggested that, contrary to the observations of Lin et al. (1999), fertile workers were ingesting and digesting pollen. They were obviously not totally dependent on jelly produced by other bees. An experiment was therefore designed in which forager bees were used as companions. Foragers are known to have very low hemolymph levels of the egg yolk protein vitellogenin (Engels, 1972; Amdam et al., 2005), and forager-aged bees show little ovary development under queenless conditions (Jay, 1967). Their use as companion bees had not been attempted earlier because their hpg do not typically secrete jelly (Huang and Otis, 1989).

The use of foragers as companion bees finally brought the desired result of infertility in the companion bees, accompanied by strong ovary development in the younger bees used as replacements for real selected workers. All eggs produced in the cages could be attributed to young workers. These results suggest that forager bees may be suitable as companions to selected workers. This means that an important objective of the present study, selective induction of fertility in specific animals within a group of queenless workers, has been achieved.

Selected workers can be valuable, either because they show rare traits or because the selection process is work intensive. Therefore, it is important to maximize the proportion of selected workers from which eggs can be obtained. More than 60% of the young workers from the

forager experiment showed fully developed ovaries, but this does not mean that all of them were egg-layers. Data on ovary development in the young animals in combination with the results of the molecular analyses of egg maternity show that between 21.7 and 56.6% of the total number of young bees from all cages must have laid eggs. The exact proportion cannot be calculated, because each cage contained three young workers, and molecular analyses of egg maternity were only done in cases in which involvement by foragers was suspected. In the most successful trial of Harris and Harbo (1991), eggs were produced from 54% of single workers from a colony selected for rapid ovary development, placed inside cages with 25 companion bees from colonies in which worker ovary development was slow. The method of Harris and Harbo is hardly feasible in practice because the type of companion bees they used is very difficult to obtain for ordinary breeders (see introduction). For against, foragers can be collected very easily during a great part of the honey bee season. One method to do this is to spray combs with a mixture of honey and water and place them in the vicinity of a bee yard. After a short while, great numbers of foragers can usually be collected from these combs by brushing them into a box.

It would of course be important to know how many eggs each laying selected worker can produce. In the study of Harris and Harbo (1991), individual workers inside groups of companion bees produced an average of 11 ± 12 (mean ± standard deviation) eggs over a period of 8 days. In the experiment involving foragers as companions, one worker was observed that laid at least 15 eggs within four days.

2.3 Evaluation of the quality of worker-laid eggs

2.3.1 Biological background

The viability and fitness of worker-laid eggs, and of larvae and drones developing from them, are evidently of critical importance to the development of methods for breeding from worker bees. Very little data are available on these points. When a strong colony in the field loses its queen and is unable to rear a replacement, high levels of worker reproduction often occur, which can lead to the production of several thousands of males before the colony breaks down (Page and Erickson, 1988). This proves that worker-laid eggs can give rise to adult drones. However, the proportions of eggs, larvae and pupae that die before reaching the adult stage are unknown.

Some data exist on the viability of the egg stage, but these data are partly contradictory. Pirk et al. (2004) found that little more than 20% of worker-laid eggs develop into larvae, far less than in queen-laid eggs (approximately 80%). Velthuis et al. (2002) found 35% hatching success for worker- and 87% for queen-laid eggs. For against, Beekman and Oldroyd (2005) found that hatching rates were on average slightly higher in eggs of workers (78%) than in eggs of queens (68%). Ratnieks and Visscher (1989) reported almost identical hatching rates for both egg types. Worker-laid eggs are known to be bigger and heavier than eggs laid by a queen. Gençer and Woyke (2006) reported that in colonies of the subspecies *A.m. caucasica,* the difference in weight amounted to 22.3%.

Almost no information exists on the viability of worker-derived larvae or pupae. Ratnieks and Visscher (1989) transferred one day-old worker-derived larvae from a queenless colony into a queenright colony and found that 73% were still present and alive after 24 hours.

Not much more is known about the properties of adult drones that are sons of workers. Queenright colonies rear drones only in cells that are slightly bigger than those in which worker larvae develop, whereas queenless colonies use both drone- and worker-sized cells. Gençer and Firatli (2005) compared the weight and sperm numbers of drones from queenright colonies (presumably stemming from queen-laid eggs) and of drones that had been reared in drone- or worker-sized cells in queenless hives. They found that drones from queenright colonies were heavier and formed larger numberes of sperms than either type of males from queenless colonies. Worker-derived drones from worker-sized cells were lightest and showed the lowest sperm counts. Unpublished data by Al Lawati et al. (personal communication) confirm these measurements. It is not clear however whether these differences result from characteristics of queen- and worker-laid eggs, or from differences in the amount and quality of brood care provided to drone larvae in queenright and queenless colonies. Despite the finding that worker-derived drones may have less sperm in their seminal vesicles (Gençer and Firatli, 2005), sperm from worker-derived drones can be used for breeding purposes, and queens inseminated with such sperm can produce fertilised eggs (Ruttner, 1983; Oldroyd and Osborne, 1999). Therefore, using sons of worker bees for breeding is possible in principle.

Figure 6 shows examples of the three types of drones compared by Gençer and Firatli, and by Al Lawati et al..

Information is lacking about all four life stages of worker offspring. Because of time limitations, only the egg stage could be studied here.

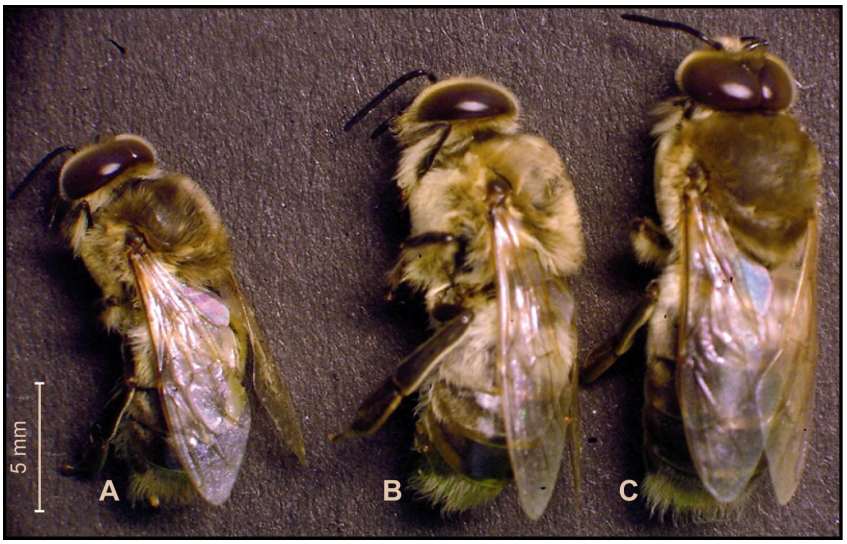

Figure 6: Habitus of queen- and worker-derived drones.

A: reared from a worker-laid egg inside a queenless colony (worker-sized cell)
B: reared from a worker-laid egg inside a queenless colony (drone-sized cell)
C: reared from a queen-laid egg inside a queenright colony (drone-sized cell)

2.3.2 Manuscript 5

Wegener, J.; Lorenz, M.W.; Bienefeld, K.

Differences between queen- and worker-laid eggs of the honey bee (Apis mellifera).

In preparation for publication in **Apidologie**

Different authors have reported hatching rates of worker-laid eggs ranging from 23% (Pirk et al., 2004) to 78% (Beekman and Oldroyd, 2005). The experiments described in manuscript 5 therefore tried to determine whether the quality of worker-laid eggs is sufficient to make breeding from workers feasible. Queen- and worker-laid eggs cannot be obtained from the same colony, because very little worker reproduction takes place in queenright colonies (Visscher, 1989). Therefore, queenright colonies were split in two, one part receiving the queen and the other part remaining queenless. The following characteristics of queen- and worker-laid eggs from such pairs of colonies were compared:

- Sensitivity towards desiccation (=hatching rates at different levels of air humidity).
- Weight and nutrient content (nutrients quantified: vitellin, total protein, lipids, free carbohydrates, and glycogen).
- The duration of embryonic development (time between egglaying and egg hatch).

Worker-laid eggs showed to be more sensitive to desiccation than queen-laid eggs. However, worker-laid eggs showed relatively high (>60%) proportions of hatching success at high levels of air humidity, and the hatching success of the two egg types did not differ under humid conditions.

Worker-laid eggs were heavier in two pairs of colonies out of four, but contained slightly smaller quantities of both lipids (two pairs out of four) and vitellin (one pair out of four). The other nutrients were always found in similar amounts in both types of eggs. Worker-laid eggs took several hours longer to develop than queen-laid eggs.

The fact that eggs from laying workers are more sensitive towards dry air may explain some of the differences between the results of earlier studies. Ratnieks and Visscher (1989) and Beekman and Oldroyd (2005), who found no difference between the hatching rates of the two egg types, both incubated them at high relative humidity. Velthuis et al. (2002), who incubated eggs at 50% rH, observed lower hatching rates in the samples collected from workers. The fact that the chorion of worker-laid eggs proved more permeable to water vapour, together with the observation that these eggs took longer to develop, led to the hypothesis that worker-laid eggs may be partly immature at the moment they are laid.

2.3.3 Achievements

Several of the results of manuscript 5 are of practical relevance to the approach of breeding from worker bees. The first is that resistance of worker-laid to dryness is low. This supports the hypothesis of Velthuis et al. (2002) that many worker-laid eggs may die from desiccation. In experiments 1 and 2 of manuscript 5, reduced hatching rates were observed at 30 and 50% rH. Inside bee colonies, air humidity may sometimes drop lower than 50% rH (Büdel, 1960). This suggests that in order to maximize hatching rates, it may be advantageous to incubate the eggs under conditions of high humidity *in vitro*. A second interesting result from manuscript 5 is that worker-laid eggs show hatching rates that are comparable to those of queen–laid eggs if air humidity is high. This means that in principle, egg viability does not pose a problem to

the prospects of producing drones from the eggs of selected workers. The third significant outcome of the study on worker-laid eggs is that on average, they may contain slightly smaller amounts of both yolk protein and lipids than eggs from queens. The differences were relatively small in both cases, especially as compared to the large inter-colony variations. As proportions of hatching larvae from worker- and queen-laid eggs were similar at high humidity, the smaller amounts of nutrients in worker-laid eggs do not appear to affect hatching success. Nevertheless, consequences for the fitness of larvae or adult drones stemming from worker-laid eggs cannot be excluded. Preliminary results from the in vitro-rearing of queen- and worker-derived larvae according to the protocol from manuscript 7 suggest that the survival rates and growth of both kinds of animals may be similar at least up to pupation (unpublished data). More data on the characteristics of worker-derived larvae, pupae and drones are clearly needed.

The reason why so little is known about the properties of worker offspring is likely to lay in the fact that until now, methods for the controlled rearing of such offspring have been lacking. Progress described in the next paragraph may stimulate research on this subject.

2.4 Rearing of worker-laid eggs into adult drones

2.4.1 Biological background

The easiest way to rear eggs of selected workers would doubtlessly be to directly introduce them into colonies, and let the nurse bees do the rest. Unfortunately, worker bees in the presence of a queen rapidly and systematically destroy worker-laid eggs (e.g. Visscher, 1989; Ratnieks and Visscher, 1989; Martin et al., 2002; Pirk et al., 2004; Beekman et al., 2007). If the queen is removed from the hive, destruction of eggs by workers continues for several weeks (Miller and Ratnieks, 2001). After this time, mass egglaying by workers starts, and the proportion of eggs that are removed by other worker bees slightly drops (Miller and Ratnieks, 2001). Some of the eggs produced during this phase are eventually reared into drones.

In queenright colonies, the proximate cue used by workers to detect worker-laid eggs is believed to be the absence of a hypothetical egg-marking pheromone of the queen (Ratnieks, 1995; Oldroyd et al., 2002). Pirk et al. (2004) suggested that an additional or alternative cause of egg removal could be the lack of viability of worker-laid eggs. Beekman and Oldroyd

(2005) contested this view by showing that killing eggs does not lead to increased removal rates.

In the resent study, extensive work has been done to protect worker offspring from destruction by other workers. Only parts of it are presented here.

2.4.2 Manuscript 6

Wegener, J.; Al Kahtani, S.; Bienefeld, K.

Collection of viable honey bee (Apis mellifera) larvae after hatching in vitro.

Accepted for publication in *Journal of Apicultural Research*

The destruction of worker-laid eggs by other workers is thought to be caused by the absence of a queen-produced pheromone adhering to the egg shell (Ratnieks, 1995). Once the egg has hatched, discrimination against worker-produced brood has been reported to stop (Ratnieks and Visscher, 1989). Therefore, a method was developed to rear viable larvae in an incubator, with the objective of transferring them to a colony afterwards. The problem with rearing larvae from eggs is that they starve within 5 hours after hatching if they do not receive food, while eggs that come into contact with larval food before hatching fail to develop (DuPraw, 1960; Collins, 2004). Also, the duration of embryonic development is variable (Collins, 2004), so that the precise moment of hatching is unpredictable.

In manuscript 6, 14 protocols are described that try to solve these problems by installing the eggs in ways that make sure that the larvae fall into food at the moment of hatching, but not before. The protocols were tested with queen-laid female eggs, because these were easier to obtain than worker-laid eggs.

The best of the tested methods consists in transferring the eggs onto wax-coated nylon strings that are strapped over food-soaked tissue paper (manuscript 6, table 1 and figure 1). Hatching larvae glide from the strings and fall into the food. Larvae only have to be collected at 12-hour intervals. With this method, 75% of eggs hatched into larvae that could be collected alive. To test whether these animals were still capable of continuing development normally, they were used to produce queens according to standard procedures (Ruttner, 1983). More than half of the eggs originally transferred to nylon strings developed into fertile queens.

The development of this protocol paved the way for the experiment described in the next paragraph, in which larvae stemming form laying workers were introduced into colonies for further rearing.

2.4.3 Manuscript 1 (continued)

Experiment 3:

Rearing of worker-laid eggs and larvae inside strong queenright colonies

In the study of Gençer and Firatli (2005), drones from queenright colonies showed a higher weight at emergence, and higher numbers of sperm than drones reared in queenless hives. In this experiment, we therefore tested different methods to introduce worker offspring into queenright colonies for rearing.

Pairs of queenright and queenless colonies were formed as in manuscript 5, and combs containing many queen- or worker-laid eggs were obtained from each of the colonies. The following methods were tested for rearing worker-laid eggs:

1. Eggs were kept in an incubator at high humidity until 48 to 72 hours old, in order to minimize the time they had to spend in the colonies before hatching. They were then transferred into queenright colonies, where they replaced queen-laid male eggs of the same age.
2. Eggs were reared into larvae in an incubator according to the protocol described in manuscript 6. They were then placed inside the queenright hives, where they replaced male larvae of the same age.
3. Eggs were left inside their native (queenless) hives, where their survival was monitored.

None of the methods for rearing worker-laid eggs yielded more than 3% drones. Rearing eggs into larvae before introducing them into the colonies did not improve their survival. The removal of the larvae was not caused by the caste of their mother, since control larvae stemming from queens were also removed. Survival of worker-laid eggs in laying worker colonies was equally low. Results of methods 1 and 2 seem to confirm the conclusion of Nonacs (2006) that queenright colonies apply strict standards for accepting male brood for rearing. These standards appear to be sticter than those applied to female larvae, since female larvae

hatched *in vitro* were accepted by rearing colonies (see manuscript 6). In conclusion, rearing offspring of selected workers inside normal queenright or queenless colonies is probably not an option, unless new methods are found to protect them from cannibalism.

2.4.4 Manuscript 7

Wegener, J.; Lorenz, M.W.; Bienefeld, K.

Successful in vitro-rearing of honey bee (Apis mellifera) drones

Submitted for publication in *Journal of Apicultural Research*

As larvae reared from worker-laid eggs inside an incubator cannot be re-introduced into colonies (Manuscript 2, experiment 3), a method was devised to rear first-instar drone larvae into adults *in vitro*. Such protocols were already available for female larvae (Peng et al., 1992; Aupinel et al., 2007), but not for male larvae.

The procedure that was developed is based on the of Peng et al. (1992) for the production of workers. It involves rearing of larvae inside the wells of tissue culture plates, which are partly filled with liquid diet (figure 7). Mature larvae are treansferred into glass vials for pupation. A new feeding regime was developed which meets the nutritional requirements of drone larvae. It consists of two different diets that are fed to larvae at different ages.

More than 50% of larvae reared according to this protocol emerged as adults. These results are far better than those obtained with any other published method for the *in vitro*-rearing of drones (Woyke, 1963; Takeuchi et al., 1972). However, 80% of the emerging animals presented malformations.

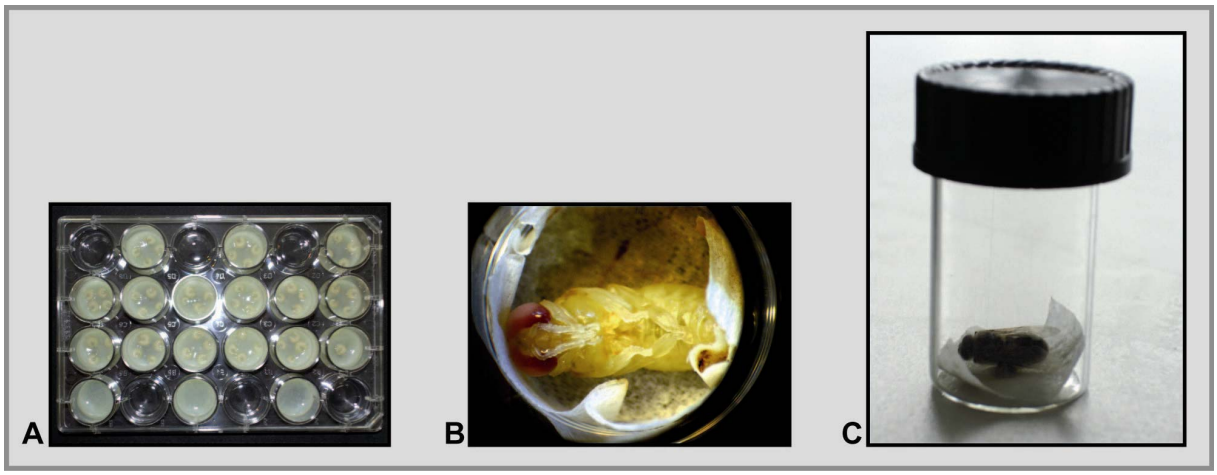

Figure 7: Steps of the protocol for *in vitro*-rearing of drones.

A: Larvae on multiwell plate.

B: Pupa inside glass vial

C: Adult drone emerged inside glass vial

2.4.5 Manuscript 1 (continued)

Experiment 4:

Acceptance of worker-laid eggs by small colonies

The *in vitro*-rearing of eggs into viable larvae (manuscript 6) is work intensive, and the results of manuscript 1, experiment 3 show that male larvae produced in this way cannot be transferred into colonies. Therefore, we tested a method to rear worker-laid eggs into larvae using specially prepared micro-colonies of approximately 1000 workers. Polaczek et al. (2000) had shown that a similar method can be used to rear diploid drones. Diploid drone larvae, like eggs of laying workers, are usually destroyed inside honey bee colonies (Woyke, 1963).

In total, 32 small colonies were used to test the influence of the presence or absence of an unmated queen, and of capped brood on the acceptance rate of added worker-laid eggs. Either freshly laid eggs (0-24 hours old) or eggs that had been incubated under conditions of high air humidity until shortly before hatching were used.

No significant difference was observed between egg acceptance by groups of colonies containing or not containing brood and/or queens. Pre-incubation of eggs before introduction led

to higher proportions of acceptance. A maximum of 20% of the eggs were reared into first instar-larvae. Although this number is rather low, the method may present an alternative to the *in vitro*-hatching of larvae.

2.4.6 Achievements

The rearing of worker-laid eggs into drones turned out to be the biggest obstacle to the use of worker offspring for breeding. Harris and Harbo (1991), in their study on methods for breeding from worker bees, did not comment on this problem. All attempts to insert worker-laid eggs into normal queenright colonies failed (experiment 3 of manuscript 2, and other experiments that are not reported here because they did not lead to publications). Because Ratnieks and Visscher (1989) had published data that seemed to suggest that eggs are more at risk of being removed than young larvae, we developed a protocol to produce viable larvae *in vitro* (manuscript 6). However, nearly all male larvae produced in this way and introduced into queenright colonies were quickly destroyed (experiment 3 of manuscript 2). It is not clear what prompted their destruction, especially as female larve produced in the same way proved to be acceptable to colonies (manuscript 6). The caste of egg mothers (queen or workers) was not the decisive factor, because queen-derived larvae produced in vitro were also removed. Although rearing of eggs inside micro-colonies only resulted in limited proportions of accepted larvae (maximum 18%), this method may be an interesting alternative to the *in vitro*-protocol if it turns out that these larvae are more acceptable for rearing inside colonies.

As no way could be found to rear worker offspring inside colonies, a method was developed to rear drone larvae into adults in an incubator (manuscript 7; figure 7). The protocol was repeated with larvae from three different colonies, and although variations occurred between the repetitions, the proportion of adults that emerged was always higher than 50%. The fact that many of the drones reared in this way presented malformations (figure 8) limits the value of the protocol in its current form. At least some of the malformations were probably due to the fact that the procedure requires daily transfers of the larvae into fresh food, which may lead to sub-lethal injuries. Aupinel et al. (2007) developed a method for the *in vitro*-rearing worker bees which makes these daily transfers unnecessary. Possibly, the combination of the diets developed in the present study with the rearing method of Aupinel et al. (2007) could help to reduce the frequency of malformations.

Queen-derived drone larvae were used for developing the *in vitro*-protocol in manuscript 7. Later however, the protocol was also validated for rearing worker-derived larvae. In this experiment, 80% of the larvae reared emerged as adults (unpublished data).

After hatching, drones have to spend approximately 11 days in the company of worker bees in order to attain sexual maturity (Ruttner, 1996). Only after this time can sperm be obtained from them. Unfortunately, the ability of drones reared *in vitro* to complete maturation could not be assessed, and their fertility could not be measured. Another disadvantage of rearing drones in an incubator is that this method is work intensive and technically demanding.

In summary, progress towards the rearing of worker-derived drones has clearly been made in the course of this study, but the last step to reach a workable and reliable protocol still has to be taken.

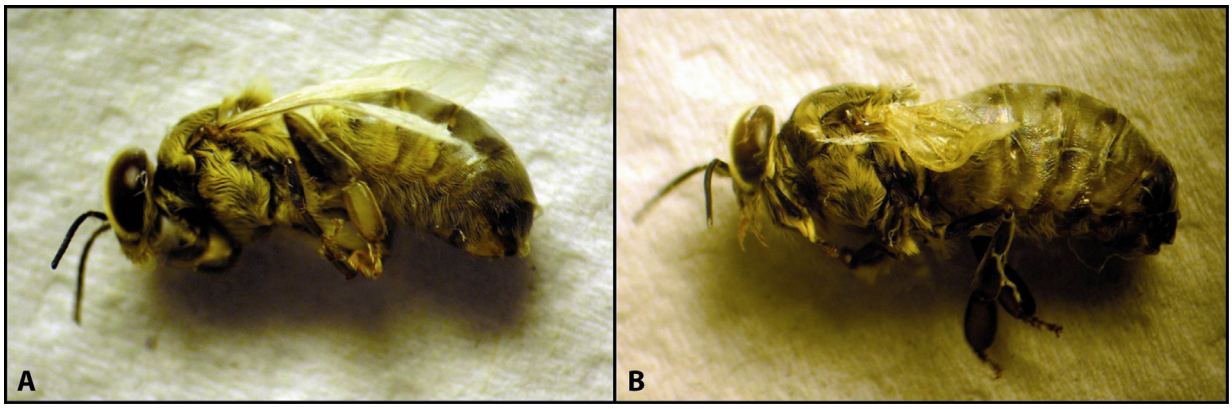

Figure 8: Drones reared in vitro according to the procedure from manuscript 7.

A: individual with normal morphology.

B: individual presenting severe malformations.

Most of the drones reared showed a morphology that lay between these two extremes.

3 General Conclusions

The aim of this study was the development of methods to produce eggs from worker bees carrying interesting traits for breeding, and to rear these eggs into fertile drones. Although great progress has been made, the objective of producing an easy and reliable protocol has not been fully achieved. A preliminary protocol is given in an appendix. It is an assembly of procedures described in the different manuscripts that form this study. In order to make the protocol intelligible without reference to the manuscripts (which will not be contained in the online version of this thesis), some of the figures from the manuscripts have been reproduced in the appendix. The protocol involves the following steps:

1. The induction of fertility of selected workers by association with foragers under queenless conditions
2. The incubation of eggs under conditions of high air humidity in an incubator up to an age of 48 – 72 hours.
3. Transfer of eggs onto wax-coated nylon strings strapped over food-drenched tissue paper (manuscript 6, figure 1) and further incubation under humid conditions or, alternatively, rearing of eggs into larvae inside micro-colonies.
4. *In vitro* – rearing of larvae into adult drones

This protocol presents several serious flaws. Firstly, it is incomplete, because no methods have been developed to transfer drones from the incubator into colonies for sexual maturation. Secondly, although the individual parts of the protocol have all been tested, the procedure as a whole has never been tried out. Thirdly, the protocol is relatively work-intensive. Finally, each step involves losses in the form of selected workers that fail to produce eggs, or of worker offspring that dies in the course of rearing. The percentages of expected losses are shown in figure 9.

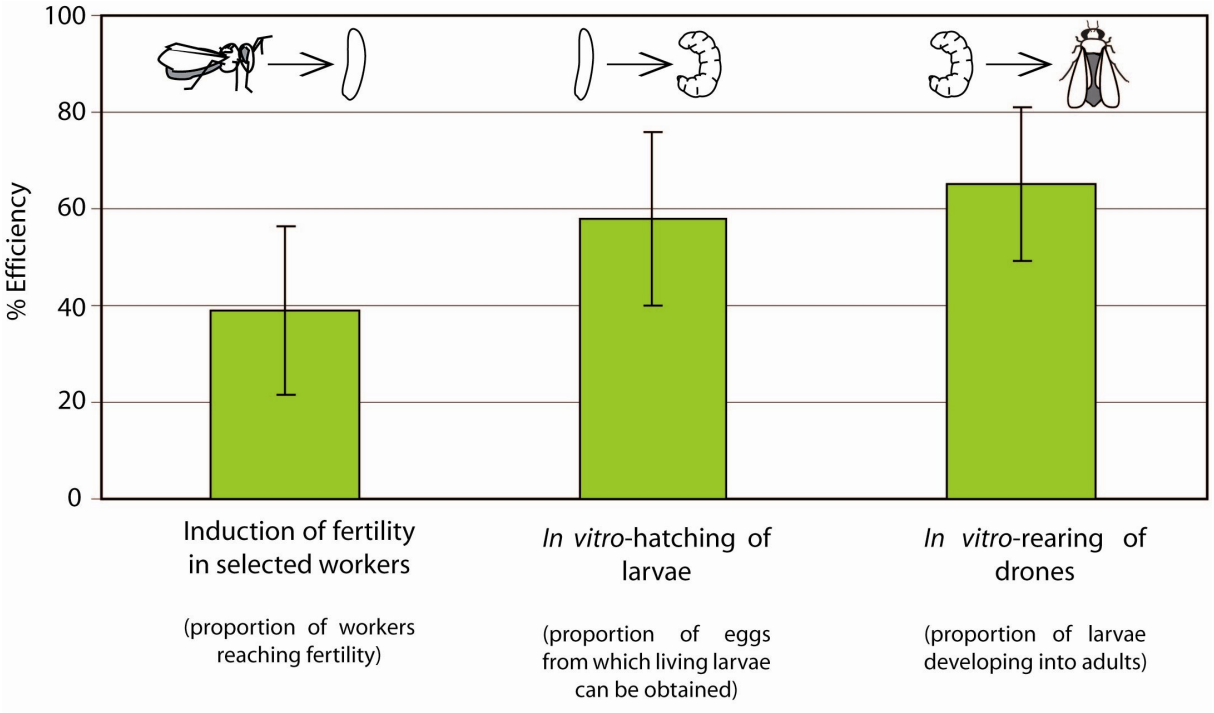

Figure 9: "Efficiency" of steps involved in production of worker-derived drones.

Step from worker to egg: Error bars give upper and lower boundaries for the estimated proportion of laying workers in the forager experiment (manuscript 1, experiment 2).
Step from egg to larva: Error bars enclose range of proportions of worker-laid eggs that could be reared into living larvae (n = 5 samples of 100 – 260 eggs; unpublished data).
Step from larva to drone: Error bars enclose range of proportion of queen- or worker-derived larvae reared into adults (n = 4 experiments; data from manuscript 7 and unpublished data).

Nevertheless, it seems likely that sons of selected workers can eventually be produced with this protocol. The central problem of inducing fertility in selected members of a queenless group of worker bees has been solved. Ways have been found to protect worker-derived eggs and larvae from destruction by other workers, by rearing them in an incubator. A method has been found to rear these larvae into adult drones.

Some of the methods developed during this study may be useful for applications other than breeding from worker bees. For instance, the procedure for rearing eggs into viable larvae inside an incubator (manuscript 6) may also be useful for rearing cryopreserved or genetically modified embryos. The protocol for the *in vitro*-rearing of drone larvae (manuscript 7) may equally be used to rear other types of larvae which would be cannibalized inside colonies. Since the method was first presented at a conference, several laboratories have announced their intention to use it.

Apart from methodological advancements, the study has directly contributed to different areas of honey bee research, including nutrition (manuscript 7), social biology (manuscripts 2 and 4), and physiology (manuscripts 2 and 5).

The main motivation for this study was the prospect of accelerating the selection of hygienic honey bees by breeding from workers. At the moment, colonies in most parts of the world have to be treated systematically against one or several brood diseases (Faucon and Chauzat, 2008; Boecking and Genersch, 2008). Selecting bees that can cope with these diseases by themselves is doubtlessly the biggest task of applied honey bee research for the coming years and decades. If breeding from worker bees can contribute to it, then the considerable investments of time and resources that are required to produce sons of selected workers may be justified.

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List of manuscripts

The following is a list of the manuscripts summarized in part 2 of the present dissertation. Because of copyright regulations, the complete articles cannot be given in the online version of the thesis. In order to partly compensate for this, a protocol summarizing the methodological advances contained in the seven manuscripts is given as an appendix (appendix 1).

1. Wegener, J. & Bienefeld, K. (in press) Methoden zur Zucht der Honigbiene unter Nutzung der Nachkommen von Arbeiterinnen. Zuechtungskunde

2. Wegener, J., Huang, Z.Y., Lorenz, M.W. & Bienefeld, K. (published online before print) Regulation of hypopharyngeal gland activity and oogenesis in honey bee (*Apis mellifera*) workers. Journal of Insect Physiology, DOI: 10.1016/j.jinsphys.2009.05.003.

3. Wegener, J., Zautke, F., Höcht, S., Köhler, B. & Bienefeld, K. (2006) Suppression of worker fertility in the honeybee (Apis mellifera) by treatment with X-rays. Journal of Apicultural Research, 45, 27-32.

4. Wegener, J, Lorenz, M.W. & Bienefeld, K. (2009) Physiological consequences of prolonged nursing in the honey bee. Insectes Sociaux, 56, 85-93.

5. Wegener, J., Lorenz, M.W., Bienefeld, K. (accepted subject to revision) Differences between queen- and worker-laid eggs of the honey bee (*Apis mellifera*). Apidologie.

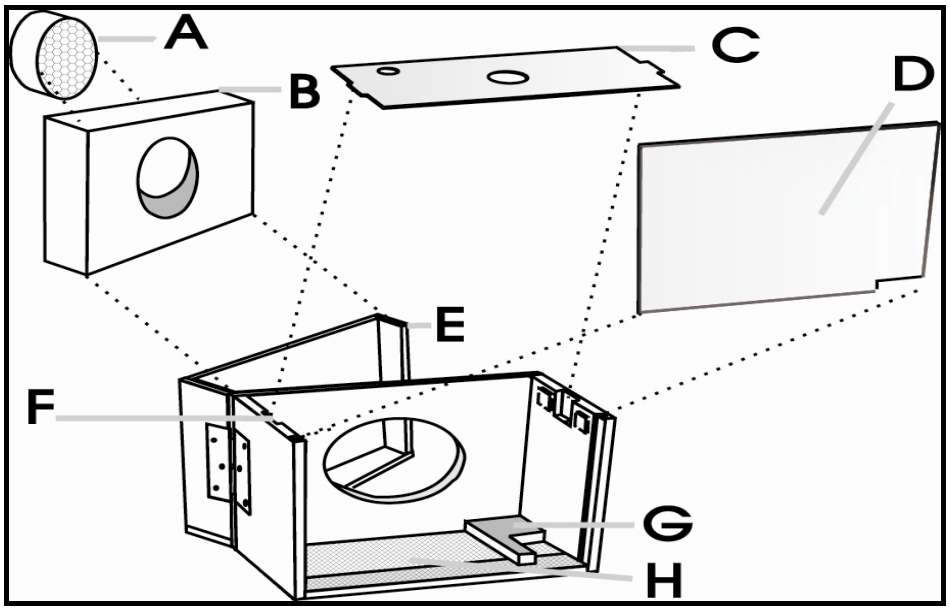
6. Wegener, J., Al-Kahtani, S., Bienefeld, K. (2009) Collection of viable honey bee (*Apis mellifera*) larvae after hatching in vitro. Journal of Apicultural Research, 48, 115-120.

7. Wegener, J., Lorenz, M.W., Bienefeld, K. (in preparation) Improved diet for the *in vitro*-rearing of honey bee drones. Journal of Apicultural Science.

Appendix: Preliminary protocol for the production of off-spring from selected worker honey bees

A. Materials and diets

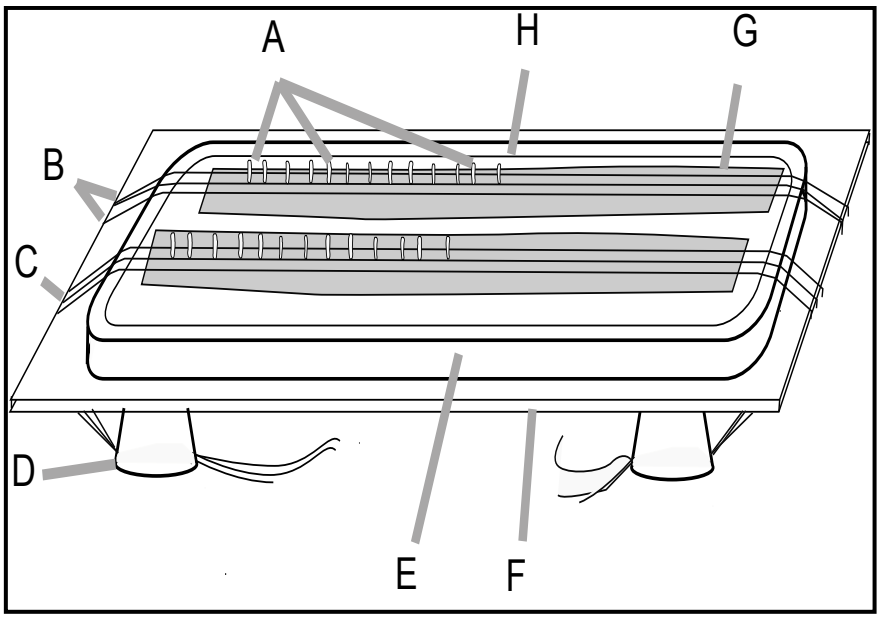
A.1 Cages for the production of worker eggs in an incubator



A: Round piece of drone comb, inserted into a piece of plastic tubing (Ø 8 cm)
B: Block of foamed material
C: Lid (acrylic glass) with holes to insert bees and water dispenser
D: Front pane (acrylic glass)
E: Hinged rear wall (plywood
F: Cage (plywood), 20 x 15 x 8 cm
G: Fitting for food trays
H: wire-mesh bottom

In order to collect eggs, the hinged rear wall (E) is slightly opened and a thin plastic slide is inserted between the comb (A) and the main body of the cage (F). The plate is held in position by wooden fittings. It closes the cage while the comb is taken out and checked for eggs.

A.2 Apparatus to avoid starvation of larvae hatching in an incubator



A: Eggs, placed on nylon string

B: Cotton strings (not required)

C: Nylon string (Ø 0.25 mm, coated with beeswax)

D: Rubber plug with incisions, screwed to F and used to fix strings

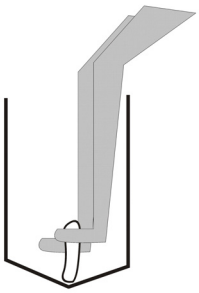
E: Lid of cell culture plate

F: Support (acrylic glass)

G: Tissue paper, soaked with larval diet A.51

H: Rim on the lid, keeping strings slightly above lid surface

A.3 Forceps for transferring honey bee eggs



This tool was described by Taber (J. Econ. Entomol. 54: 247-250) and allows the transfer of eggs without crushing them. It can be produced from a pair of flat forceps (the type used for handling postal stamps). The tips should not touch each other when the forceps is squeezed. Rather, they should leave open a gap that is large enough to enclose the egg and only slightly squeeze it. The picture shows how an egg is seized by the forceps.

A.4 Diet for caged workers

Per 113 g:

60 g powdered sucrose

40 g ground pollen (fresh, not dried)

13 ml water

Diet can be stored for 1 week at 4°C.

A.5 Diets for *in vitro*-rearing of drones

A.51 Diet for young larvae (up to about 108 hours of age)

Per 100 g:

66.6 g royal jelly

3 g fructose

3 g glucose

27.4 ml sterile water

A.52 Diet for older larvae (> 108 hours of age)

Per 100 g:

50.0 g royal jelly

6.0 g glucose

14.0 g glucose

30 ml sterile water

Diets can be stored for one week at 4°C.

A.6 Other materials

- Incubator (temperature regulation ± 0.2°C)
- Styrofoam hive boxes for mating nuclei (Friedrich Wienhold, Germany)
- Unmated queens and young worker bees (if technique C.2 is chosen for egg rearing)
- Tissue culture plates (number 602160, Greiner BioOne, Germany)
- Disposable plastic syringes (1ml) for handling larval diet
- Round glass vials for pupation (e.g. number X659.1, Carl Roth, Germany)
- Loosely fitting plastic caps for the pupation vials (inner diameter approximately 3 cm)

B. Procedure to obtain eggs from selected workers

Ideally, selected workers should be 7-9 days old at the moment of caging.

- Collect forager bees as companions to selected workers. To do this, install combs sprayed with a mixture of honey and water near an apiary and wait until forager bees arrive to collect the honey. Then, brush forager bees into a box with air holes.
- Add a piece of bee candy (a stiff dough of honey and powdered sucrose in the ration 1:5) to the cage and place inside a dark and quiet room for two days
- For every three selected workers, assemble one cage (A.1), and equip it with one tray (= Lego®-stone) of diet (A.4), one tray of liquid honey, and a filled water dispenser.
- Place groups of 30 forager bees into each cage
- Per cage, take three selected forager bees and briefly dip them into liquid honey. Insert them into the cage. The honey is meant to facilitate acceptance of selected bees by the foragers.
- Place cage inside incubator at 34°C, 50 – 70% relative humidity.
- Change food and water every two days.
- From about the sixth day onwards, check combs for the presence of eggs. If eggs are found, remove the comb that contains the eggs and replace it by a fresh one.
- If egg production is to be continued for longer periods, forager bees should be changed for fresh ones every two weeks.
- After the end of egg production, check ovary development in foragers. A description of the procedure for dissection of ovaries can be found in Dade (Anatomy and dissection of the honeybee. IBRA, London, 1962). If one or several of the foragers present ovaries with elongated oocytes, it has to be doubted whether the eggs obtained really stem from the selected workers.

C. Rearing of eggs into first instar larvae

Two alternative methods are given for this step. Method one yields higher numbers of living larvae, but the vitality of larvae produced by method 2 might be slightly higher.

C.1 In vitro – technique

- Place the round combs containing the eggs in an incubator at 34°C and 90 – 95% relative humidity. Incubate for approximately 36 hours.
- For every 60 eggs, prepare approximately 40 ml of diet A.51 and dilute it with sterile water (1 part of water, four parts of diet)
- Prepare strips of tissue paper 2 x 8 cm
- Prepare wax-coated nylon strings by heating a small quantity of wax on the blade of a knive and tearing the string through the liquid wax.
- Place the lid of the tissue culture plate ("E" n figure A.2) on the support ("F" in figure A.2), and place a few ml of diluted diet on the lid using a pipette. Put four tissue paper strips on top of each other and place them on the puddle of diet.
- Cover the strips with diluted diet until they are completely soaked. However, all the liquid should be absorbed by the paper. No puddles should remain on the paper.
- Strap wax-coated nylon string over the moist paper and fix it by quenching in the incisions in the rubber stoppers ("D" in A.2).
- Using special forceps (Taber, 1961), remove the eggs from the combs and place them on the strings in an upright position. Some people find it easier to do this under a binocular.
- Incubate at 34°C, >95% relative humidity.
- Collect larvae from the tissue paper every 12 hours using a standard grafting tool.

C.2 Technique using micro-colonies

- Set up micro-colonies in styrofoam hive boxes for mating nuclei. Into each colony, add the following:
    - 2 Empty combs
    - 2 combs of honey and pollen
    - An unmated queen in a wire mesh cage
    - 100 – 150 g of young worker bees
- Place micro-colonies in a dark room overnight.
- Install colonies in the apiary.
- Place the round combs containing the eggs from step B. in an incubator at 34°C and 90 – 95% relative humidity. Incubate for approximately 36 hours.
- For each egg-containig comb from B., take out one empty comb from one of the micro-colonies. With a hot knive, produce a hole in the comb in which the round comb with eggs can be inserted. Insert the round comb.
- Place the comb from the micro-colony (containing the round comb) back into its hive.
- Collect larvae after 24 – 36 hours.

D. *In vitro* - rearing of larvae into drones

- Prepare diet A.51 and warm it in an incubator (34.5°C) for one hour.
- On the tissue culture plate (24 wells), fill 6 wells almost to the brim with sterilized water.
- Fill additional wells with 300 µl each of diet A.41, using a disposable syringe. On the first day of *in vitro*-rearing, larvae are still small, so that 10 fit into one well. As larvae grow, this number should be reduced. Before defaecation, each larva should have a well on its own.

- Place larvae into wells. When larvae are still small, care should be taken not to submerge them, because they may drown.
- Place plate in the incubator at 34.5°C, >95% relative humidity.
- Transfer larvae into fresh, pre-warmed food daily.
- From day 5 of in vitro-rearing onwards, use diet A.52 instead of A.51.
- Prepare a sufficient number of pupation vials (sterile), by placing a piece (approx. 2 x 2 cm) of sterile, lint-free paper on the bottom.
- Transfer larvae that show one or several of the following signs to pupation vials:
    - Appearance of clear crystals (uric acid) in the diet
    - Appearance of brownish/yellowish faeces
    - Larva spinning silk
- Larvae in pupation vials can be incubated under the same conditions as tissue culture plates. They should be left undisturbed for at least five days.
- As soon as the first pupae begin to move slightly, place a small quantity of honey on the inte-rior side of the cap. From now on, check vials for emerged adults at 12 hour - intervals.

Versicherung über Selbständigkeit der erbrachten Leistung

Hiermit erkläre ich, dass ich meine Dissertation selbständig und ohne unerlaubte Hilfe angefertigt habe, und dass sie bisher weder ganz noch in Teilen einem Promotionsverfahren zugrunde lag.

Jakob Wegener